ISOLATION, CONFIRMATION AND SPECIFIC DETECTION OF *Cronobacter sakazakii* FROM MILK AND MILK BASED PRODUCTS

SYNOPSIS

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Doctor of Philosophy

In

Zoology

Submitted by

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INTRODUCTION

Cronobacter sakazakii is an opportunistic, gram-negative, facultative, nonspore-forming, motile bacteria of the Enterobacteriaceae family. It is an emerging food borne pathogen that causes clinical symptoms including meningitis, bacteremia, brain abcess, septicemia, meningoencephalitis, and necrotizing enterocolitis in infants and neonates (Hunter and Bean 2013; Lai 2001). It is responsible for high fatality rate (up to 80%) in infected neonates (Fakruddin *et al*., 2013). The International Commission of food Microbial Specifications (ICMS) in 2002 characterized *Cronobacter sakazakii* as a "severe hazard that causes potentially life threatening chronic sequeale".

CLASSIFICATION

Domain : Bacteria

Phylum : Proteobacteria

Class : Gammaproteobacteria

Order : Enterobacteriales

Family : Enterobacteriaceae

Genus : *Cronobacter*

Species : *sakazakii*

Binomial name : ***Cronobacter sakazakii*** (Farmer *et al.,* 1980)

Being ubiquitious in nature, it can be easily isolated from food production environments and households (Kucerova *et al*., 2011). It has been commonly detected in various foods such as freshly cut fruits, ready to eat food, dry powdered food, sprouts, fruits and vegetable juices etc (Pluta *et al*., 2017). It has also been found in natural environment such as water, soil, dried meat, dairy products, other beverages and in powdered infant formula (Friedmann 2007).

Cronobacter sakazakii is fairly resistant to dry and osmotic stress conditions tolerating broad range of growth temperatures, antibiotic resistance and biofilm formation facilitates this bacteria to survive in hostile conditions (Breeuwer *et al*., 2003).

Due to these factors, infections caused by this bacterium are a great concern in immunocompromised, premature births, low weight individuals, and neonates as they are the highly vulnerable groups (Henry and Fouladkhah 2019).

Earlier various methods to detect *Cronobacter sakazakii* have been used which include biochemical tests, chromogenic assays, PCR (Polymerase Chain Reaction), 16S rRNA sequencing (Jaradat *et al*., 2009), Immunomagnetic beads, Immunoassays, Immunopolisome Based Assay (Shukla *et al.,* 2016), Enzyme Linked Immunosorbent Assays (Kong *et al*., 2015 and Li *et al*., 2013), plate counts (Pei *et al.,* 2016), electrochemical methods (Hu *et al*., 2015) and loop mediated isothermal amplification (Fan *et al*., 2012) etc. but these methods are time consuming, costly, have low accuracy and are also complex. Therefore, there is an immediate need to develop a rapid, cost effective and simple method to detect *Cronobacter sakazakii* with high senitivity to decrease food contamination and improve human health. An alternative and promising method for quick and reliable detection of *Cronobacter sakazakii* in food is the use of Aptamers.

Aptamers are single stranded nucleic acids (DNA or RNA) of random 30 to 100 nucleotide sequences isolated *in vitro*, which have the ability to bind to various targets such as proteins, small inorganic and organic molecules, metal ions and even the whole cells with high affinity and specificity. In 1990 the first apatamers were designed (RNA aptamer) against bacteriophage T4 DNA Polymerase (Tuerk and Gold 1990) and various organic dyes (Ellington and Szostak 1990).

Amongst the different types of biosensors, aptamers have unique properties which include low immunogenicity, small size, flexibilty and chemical stability (Zhou *et al*., 2010 and Han *et al*., 2010). They have the ability to change their shape and achieve specific 3D configuration such as pseudoknots, G- quadruplexes, stem loops, triplexes which allow them to bind to different target molecules and form stable and specific complexes with high affinity and specificity (Bing *et al*., 2017; Famulok and Mayor 2000).

Aptamers are designed in *vitro* by a process known as Systematic Evolution of Ligands by Exponential Enrichment (SELEX) which are evolved from Random DNA library (RDL) by repetitive binding of oligonucleotide to target molecules followed by partitioning , amplification by PCR and purification (Tuerk and Gold 1990; Liu and Zhang 2015).

For bacterial specific aptamers, Whole CELL-SELEX is used commonly to select aptamers which bind to surface molecules specifically on the target bacteria (Davydova *et al*., 2016 and Sola *et al*., 2020). Cell specific aptamers can be developed without any previous knowledge about cell surface

proteins or target molecules on the target cells (Ohuchi 2012).

Centrifugation Based Partitioning Method (CBPM) is another rapid technique to isolate bacterial specific aptamers which excludes repetitive enrichment steps that are essential in SELEX and hence makes this method less time consuming and increases aptamer affinity (Kim *et al*., 2020).

It is essential to develop rigorous control measures to reduce the risk of contamination and infection due to the severity of infections caused by *Cronobacter sakazakii.* Antibiotic therapy is commonly used to prevent *C.sakazakii* infections in humans. The inclusion of probiotics may prevent the infection by competitive exclusion and aggregation with pathogens. It has also been studied that probiotic strains have inhibitory effect on adhesion capacity of C. *sakazakii* to intestinal cells (Collado *et al.,*2008). Many naturally occuring extracts found in plants, herbs, and spices inhibit *Cronobacter* activity (Chauhan *et al.,*2020).

The present proposed study aims to detect and confirm *Cronobacter sakazakii* as rapidly as possible in milk and milk based products and to evaluate its control strategies. Further, an attempt will be made to formulate the most effective means to prevent its contamination and infection.

REVIEW OF LITERATURE

The taxonomic position of a new species of Enterobacteriaceae, previously known as "yellow-pigmented *Enterobacter cloacae*", was established as *Cronobacter sakazakii.* It was isolated from clinical specimens such as sputum, faeces, wounds, and also from food, physician's stethoscope and uninoculated blood culture bottle (Farmer *et al*., 1980). Eight cases of sepsis and meningitis in new borns, in Netherlands were reported due to *Cronobacter sakazakii* out of which 2 patients had necrotizing enterocolitis and meningitis together (Muytjens *et al*., 1983). Brain abcess in neonates due to *Enterobacter sakazakii* and the role of CT scan and MRI in its diagnostic work was examined by Burdette and Santos (2000). It was found that the use of powdered infant milk product in newborns caused infections like enterocolitis and meningitis due to contamination with *Cronobacter sakazakii* (Himelright *et al*., 2001). Infections among neonates, infants, children and adults caused by *Enterobacter sakazakii* were further reported by Lai in 2001.

Various biosensors to detect pathogenic bacteria depending on different biological elements and transducers were discussed by Ivnitski *et al.,* (1999). They also focused on methods to improve the

analysis time and sensitivity of biosensors. Lehner *et al*., (2004) developed a new PCR system to detect *Enterobacter sakazakii* strains from different sources by phylogenetic analysis of the new full-length of 16s rRNA gene sequence data of thirteen strains from food, environment and human origin. Later Nair and Venkitanarayanan in 2006 concluded that cloning and sequencing of ompA gene and developing ompA targeted PCR can be used as a rapid tool to detect *Cronobacter sakazakii* in PIF. *Cronobacter sakazakii* was identified and confirmed from infant foods, herbs and environment samples using biochemical tests, chromogenic assays, PCR and final confirmation was done by 16S rRNA sequencing by Jaradat *et al.,* (2009).

Further studies to improve detection techniques were used to detect *Staphylococcus aureus* by developing a panel of ssDNA aptamers by whole bacterium based on SELEX procedure and proved that panel of five selected aptamers showed high specificity and affectivity against the recognition of different strains of *Staphylococcus* (Cao *et al*., 2009). In 2010, Sefah *et al.*, developed the Cell-SELEX technique (Systematic Evolution of Ligands by Exponential Enrichment) to develop DNA aptamers that can bind to the cell of interest. Later in 2012, Ohuchi discussed the advantages of CELL SELEX technology over the SELEX technology to develop aptamers and stated that cell specific aptamers can be developed without previously knowing cell surface molecules on the target cells. In the same year Marimuthu *et al*., stated the use of ssDNA production in development of DNA aptamer because of its higher stability than RNA and reviewed different methods in SELEX to produce ssDNA from dsDNA followed by PCR amplification step.

The potential of ELISA technique for rapid and fast detection of *Cronobacter sakazakii* was employed by Sunhyun *et al*., (2012). They detected *Cronobacter sakazakii* by the development of sandwich enzyme-linked immunosorbent assay in which a polyclonal antibody is produced against C.*sakazakii.*

The applications of aptamers in research and food safety were summarized and analyzed by Liu and Zhang in 2015. They also discussed the advantages of aptamers over existing methods of biosensors in terms of low limit of detection and high sensitivity. Screening technologies of various aptamers were also reviewed. The modification of SELEX were stated by Singh and Pandey in 2016. They discussed CELL SELEX where whole live cells are used as targets, its applications in the field of diagnostics

and targeted therapy and its potential to discover biomarker in order to detect diseases and for therapeutic purposes. A convinient and sensitive two stage label free aptasensor was developed for colorimetric detection of *C sakazakii* in 2016 by Kim *et al*., in powdered infant formula in which aptamer is depleted by C sakazakii from test solution and the reduction of aptamer causes change in colour from red to purple which is induced by aggregation of gold nanoparticles in salt. Further Dunn *et al.*, (2017) analysed aptamer discovery and technology, its key applications and also discussed the challenges that have impeded the success of aptamers in practical applications. They had also suggested the various chemical modifications

in aptamers to improve the characteristics such as stability and activity.

The contribution of DNA sequence based methods and current knowledge of genus were reviewed by StephenJ Forsythe in 2018 to improve identification procedures, detection methods, genotyping scheme and genomic analysis. It also covered the occurrence of *Cronobacter* spp in food ingredients, finished products and food manufacturing environment.

Isolation of aptamer by non SELEX method was discussed by Kim *et al*., (2020) which includes the omission of repeated rounds of binding, incubation, seperation and amplification in SELEX technique.Serial centrifugation, one time seperation and amplification by PCR of bounded DNA to target cell allows aptamer isolation within shorter time.

All the cases of meningitis and blood stream infections among infants that were reported to the Centre for Disease Control and Prevention and in the literature from 1961 to 2018 were reviewed and the need to promote safer ways for infant feeding especially among the neonates was emphasized (Strysko *et al*., 2020).

OBJECTIVES

- To isolate *C.sakazakii* from milk and milk-based products .
- Biochemical and molecular characterization of *C.sakazakii*.
- Designing of *C.sakazakii* specific aptamer and its screening.
- Detection of *C.sakazakii* using aptamers in milk and milk based food samples.
- Control of *C.sakazakii* using probiotics, antibiotics and natural extracts.

PROPOSED METHODOLOGY

- Creating an aseptic environment for all the cultures using standard culture methods.
- Isolation of *C.sakazakii* from milk and milk-based products using selected media like Violet Red Bile Glucose Agar (VRBGA).
- Characterization of the isolates and the standard Microbial Type Culture Collection (MTCC) culture by various biochemical tests like methyl red, Voges-Proskauer (VP) test, Citrate test and Indole test (Jaradat *et al.*, 2009).
- Confirmation of *C.sakazakii* isolates by 16S rRNA sequencing (Jaradat *et al.*, 2009).
- Development of *C.sakazakii* specific ssDNA aptamer using CBPM technique with the RDL and its primers (Kim *et al*., 2020).
- Amplification by PCR, gel electrophoresis, Cloning, Sequencing and *in vitro* synthesis of Aptamer. (Kim *et al*., 2020)
- Detection of *C.sakazakii* with dye labeled aptamer using fluorescence spectrophotometer.
- Control by probiotics, antibiotics and natural extracts.

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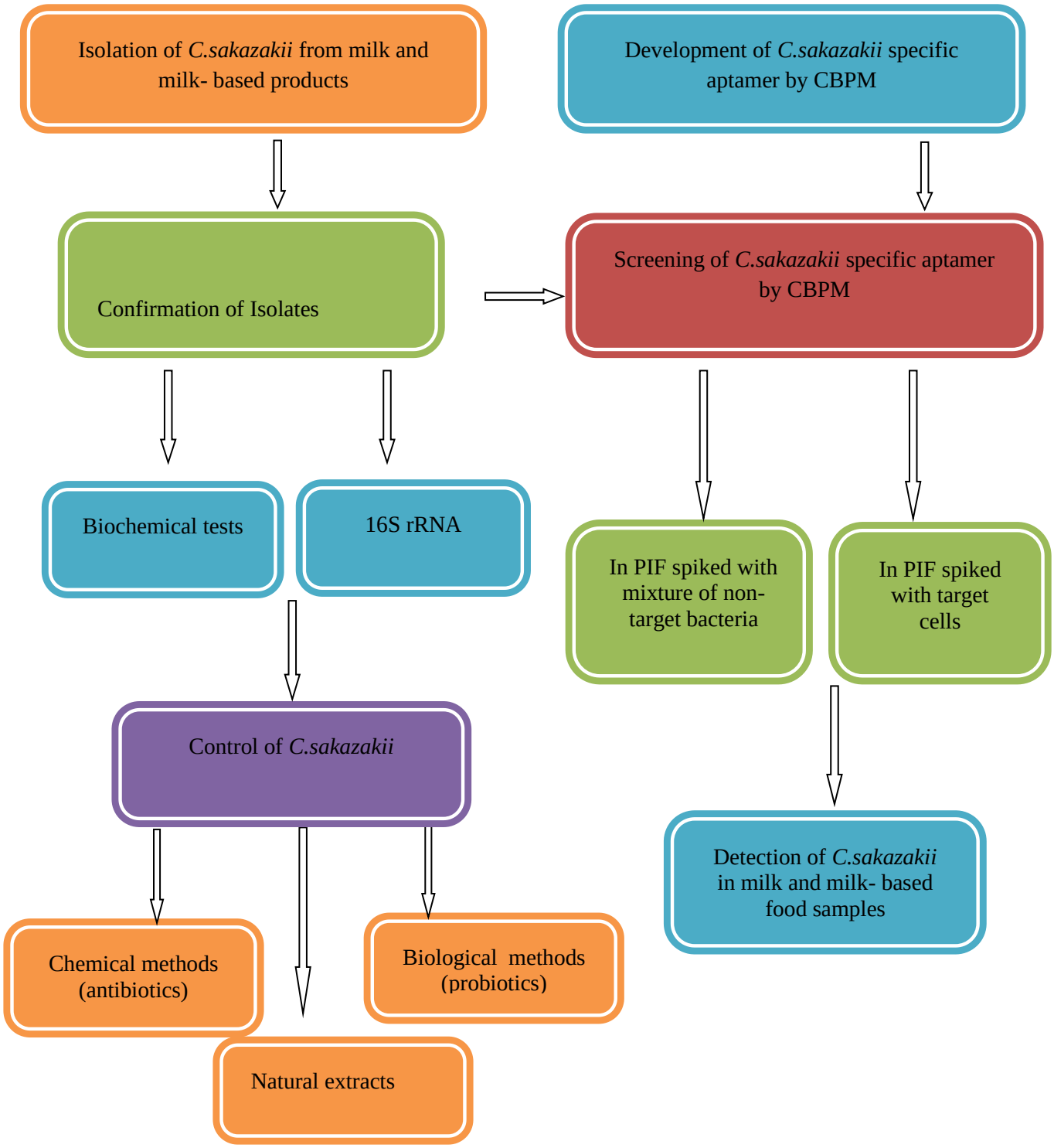
APPENDIX

WORK PLAN


Isolation of *C.sakazakii* from milk and milk- based products
Development of *C.sakazakii* specific aptamer by CBPM
Confirmation of Isolates
Screening of *C.sakazakii* specific aptamer by CBPM
Biochemical tests
16S rRNA
In PIF spiked with mixture of non-target bacteria
In PIF spiked with target cells
Control of *C.sakazakii*
Detection of *C.sakazakii* in milk and milk- based food samples
Chemical methods (antibiotics)
Biological methods (probiotics)
Natural extracts